

Tyrosine Phosphorylation Regulates the Adhesions of Ras-transformed Breast Epithelia

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Abstract. Transformed epithelial cells often are characterized by a fibroblastic or mesenchymal morphology. These cells exhibit altered cell–cell and cell–substrate interactions. Here we have identified changes in the adhesions and cytoskeletal interactions of transformed epithelial cells that contribute to their altered morphology. Using MCF-10A human breast epithelial cells as a model system, we have found that transformation by an activated form of ras is characterized by less developed adherens-type junctions between cells but increased focal adhesions. Contributing to the modified adherens junctions of the transformed cells are decreased interactions among β -catenin, E-cadherin, and the actin cytoskeleton. The ras-transformed cells reveal elevated phosphotyrosine in many proteins, including β -catenin and p120 Cas. Whereas in the normal cells β -catenin is found in association with E-cadherin, p120 Cas is not. In the ras-transformed cells, the situation is reversed; tyrosine-phosphorylated p120 Cas, but not

tyrosine-phosphorylated β -catenin, now is detected in E-cadherin complexes. The tyrosine-phosphorylated β -catenin also shows increased detergent solubility, suggesting a decreased association with the actin cytoskeleton. p120 Cas, whether tyrosine phosphorylated or not, partitions into the detergent soluble fraction, suggesting that it is not tightly bound to the actin cytoskeleton in either the normal or ras-transformed cells. Inhibitors of tyrosine kinases decrease the level of tyrosine phosphorylation and restore a normal epithelial morphology to the ras-transformed cells. In particular, decreased tyrosine phosphorylation of β -catenin is accompanied by increased interaction with both E-cadherin and the detergent insoluble cytoskeletal fraction. These results suggest that elevated tyrosine phosphorylation of proteins such as β -catenin and p120 Cas contribute to the altered adherens junctions of ras-transformed epithelia.

NORMAL epithelia are composed of highly organized sheets of interacting cells that are often poorly motile. By contrast, the morphology and migratory behavior of invasive carcinoma cells are more characteristic of fibroblasts or mesenchymal cells (reviewed in Birchmeier and Birchmeier, 1993). A similar change in morphology and behavior occurs during normal development as epithelia give rise to mesenchymal cells, for example, during neural crest development (Thiery et al., 1982). In a process known as transdifferentiation, the reverse phenomenon occurs, in which mesenchymal cells convert to epithelia (e.g., during kidney development) (Ekblom, 1989; Gumbiner, 1992). This transition between epithelial and mesenchymal morphologies can be governed by the level of protein tyrosine phosphorylation (Volberg et al., 1991, 1992; Schmidt et al., 1993). Epithelial cells transformed by the v-src tyrosine kinase generally lack orga-

nized cell junctions and acquire a more mesenchymal morphology (Matsuyoshi et al., 1992; Volberg et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993). Growth factors such as EGF or hepatocyte growth factor that elevate tyrosine phosphorylation in responsive cells induce a similar change in morphology and lead to the dissociation and scattering of epithelial cell colonies (Stoker et al., 1987; Weidner et al., 1991; Shibamoto et al., 1994).

The acquisition of a mesenchymal morphology often is characterized by decreased organization of cell junctions between adjacent epithelial cells. For example, the stability of adherens-type junctions (zonulae adherentes) is disrupted in cells that have undergone transition from epithelial to mesenchymal tissues (Birchmeier and Birchmeier, 1993; Schmidt et al., 1993). Adherens-type junctions are composed of a complex of cell surface and cytoskeletal elements which stabilize cell–cell adhesion in normal epithelia (Geiger and Ginsberg, 1991; Geiger and Ayalon, 1992; Tsukita et al., 1992). Members of the cadherin family are enriched within these structures and promote adhesion through homophilic interactions between adjacent cells (Takeichi, 1990; Geiger and Ayalon, 1992). The cytoplas-

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mic domain of the cadherins interacts with a family of molecules known as catenins, of which three are presently known: α , β , and γ catenin (Nagafuchi et al., 1993). The catenins interact in an as yet unidentified way with structural components of the actin cytoskeleton (Nagafuchi et al., 1993; Stappert and Kemler, 1993). The cadherins, catenins, and associated cytoskeletal proteins together form the adherens-type junction. These junctions encircle the apical perimeter of epithelial cells and stabilize the interactions of adjacent cells within epithelial sheets.

During the transition from epithelial to fibroblastic morphology, the reduction in adherens junctions is accompanied by an increase in focal adhesions, sites of cell adhesion to the extracellular matrix (Burrige et al., 1988). Focal adhesions and adherens junctions share certain cytoskeletal proteins, such as vinculin and α -actinin, although significant differences in the structure and composition of these two structures have also been noted (Geiger and Ginsberg, 1991). For example, the primary cell adhe-

sion molecule found in focal adhesions is generally a member of the integrin family, whereas in adherens junctions it is a cadherin (reviewed in Burrige et al., 1988). Similarly, distinct cytoplasmic components are associated with the two types of junctions: the catenins with adherens junctions (Nagafuchi et al., 1993), but talin, paxillin, and focal adhesion kinase (FAK)¹ in focal adhesions (Burrige and Connell, 1983; Geiger et al., 1985; Turner et al., 1990; Schaller et al., 1992). Many lines of evidence indicate that both focal adhesions and adherens junctions have a role in signal transduction. Both types of adhesion are sites of elevated tyrosine phosphorylation and both structures are also associated with a number of tyrosine kinases (Maher et al., 1985; Tsukita et al., 1991; Volberg et al., 1991, 1992; Burrige et al., 1992). In particular, the FAK tyrosine ki-

1. Abbreviations used in this paper: FAK, focal adhesion kinase; RIPA, Tris buffer containing 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS; TX, Tris-buffer containing 1% Triton X-100.

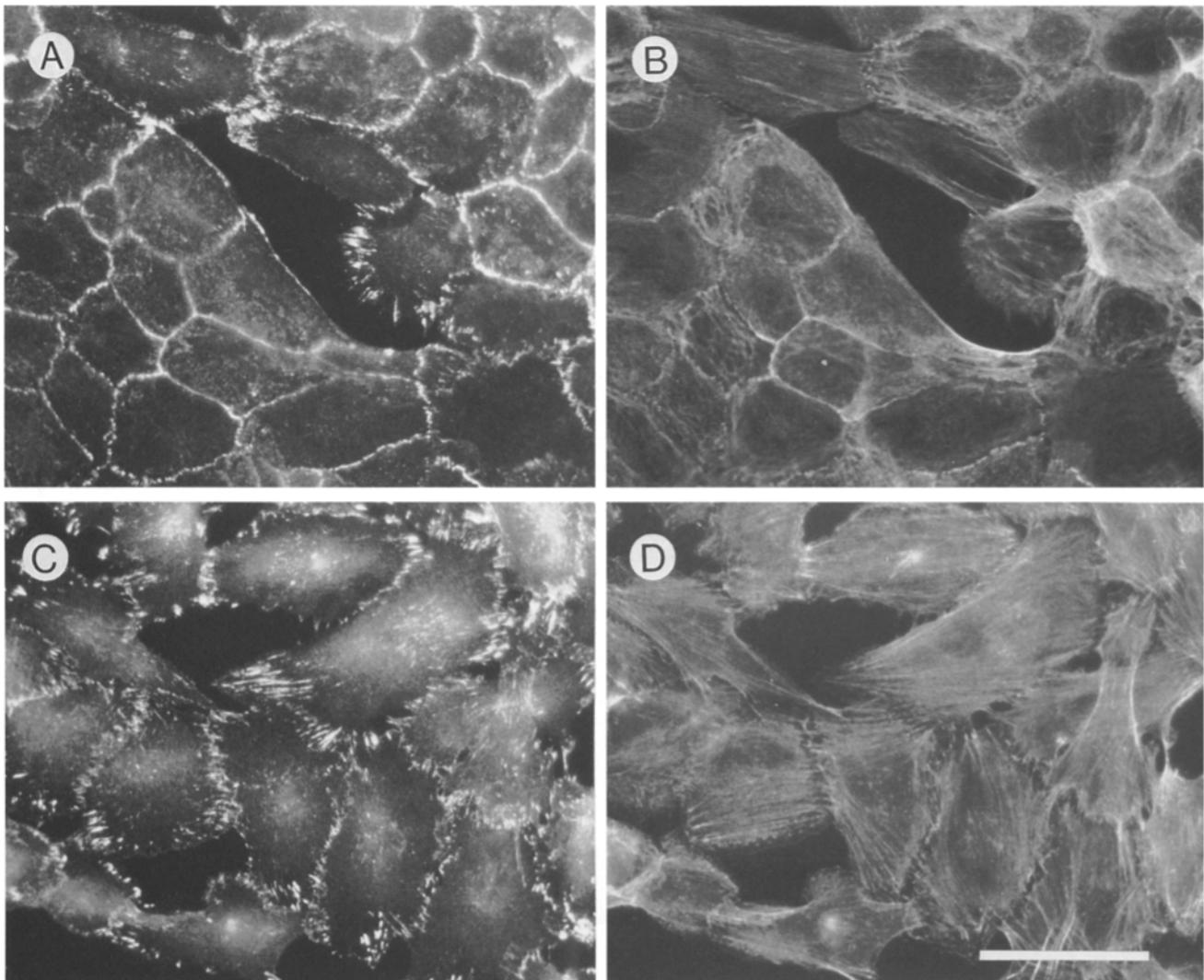


Figure 1. Transformation of MCF-10A cells with activated ras induces a reorganization of the actin cytoskeleton. MCF-10A cells, grown on coverslips in culture media overnight, were stained for vinculin (A and C) and actin (B and D). Double-labeling was used to assess the organization of the actin cytoskeleton of normal (A and B) and ras-transformed (C and D) cells. Note the increased focal adhesions and stress fibers of the ras-transformed cells. Bar, 50 μ m.

nase has been identified in focal adhesions (Hanks et al., 1992; Schaller et al., 1992), whereas members of the Src family of tyrosine kinases have been identified in adherens-type junctions (Tsukita et al., 1991). One protein initially identified as an *src* substrate is the p120 Cas molecule (Reynolds et al., 1989; Kanner et al., 1990). The cloning of Cas revealed sequence similarity to the cadherin-binding proteins β -catenin (*armadillo*) and γ -catenin (plakoglobin) (Reynolds et al., 1992; Peifer et al., 1994). Recently, Cas was found to exist in multiple isoforms and is capable of associating with E-cadherin within cell-cell contacts (Reynolds et al., 1994).

With carcinoma cells, changes in cellular adhesions often reflect a more invasive phenotype. For example, human breast epithelial cells transformed by oncogenic ras generally display reduced cell-cell adhesions and increased cell migration (Basolo et al., 1991; Ochieng et al., 1991; Miller et al., 1993). However, it is presently unclear how activated ras mediates these changes in cell behavior. Our laboratory has been interested in the regulation of the assembly and disassembly of cell adhesions. We have found previously that the formation of focal adhesions requires tyrosine phosphorylation (Burrige et al., 1992; Romer et al., 1994). To examine the regulation of cell-matrix and cell-cell adhesions in epithelial cells, we obtained two variants of the

MCF-10A human breast epithelial cell line (Soule et al., 1990). One variant of this line was created by stable transfection of the wild-type H-Ras gene and has been shown to retain a normal morphology and phenotype (Basolo et al., 1991). In contrast, another variant transfected with an oncogenic (12-Val) form of H-Ras shows the hallmarks of a transformed cell in both in vitro and in vivo assays. The ras-transformed cells are more invasive in vivo and display enhanced migratory activity in chemotaxis and chemokinesis assays in vitro (Basolo et al., 1991; Ochieng et al., 1991; Miller et al., 1993). Parallel with a change from an epithelial to a more fibroblastic morphology, we find that the ras-transformed cells show altered adherens junctions and increased focal adhesion formation. In the normal cells, E-cadherin is prominent in the adherens junctions, whereas it appears to be in less organized junctions in the transformed cells. In the normal cells, E-cadherin is associated with α , β , and γ -catenins and is linked tightly to the actin cytoskeleton. Upon transformation with oncogenic ras, E-cadherin shows decreased binding to β -catenin and increased association with p120 Cas. In the ras-transformed cells, an elevation in tyrosine phosphorylation is seen in many proteins, including β -catenin and p120 Cas. Inhibition of tyrosine kinase activity abolishes the tyrosine phosphorylation of β -catenin and restores its binding to E-cadherin.

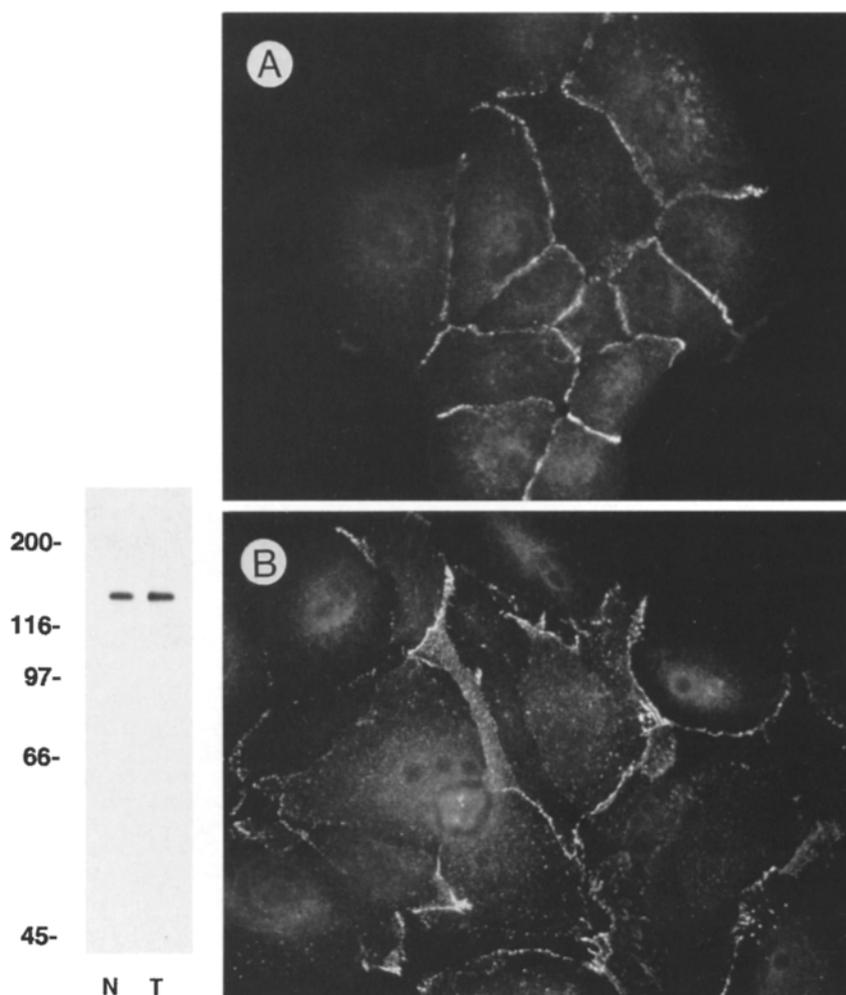


Figure 2. Cadherin expression and localization in normal and transformed MCF-10A cells. The expression of E-cadherin was assessed by immunoblotting (*left panel*). Monolayers of normal (*N*) and ras-transformed (*T*) cells were extracted with a RIPA buffer and blotted with E-cadherin-specific antibodies. The localization of E-cadherin (*right panel*) in normal (*A*) and transformed (*B*) cells was assessed by immunofluorescence microscopy. Cells were grown on coverslips in culture media for at least 24 h before immunostaining with cadherin-specific antibodies. Bar, 50 μ m.

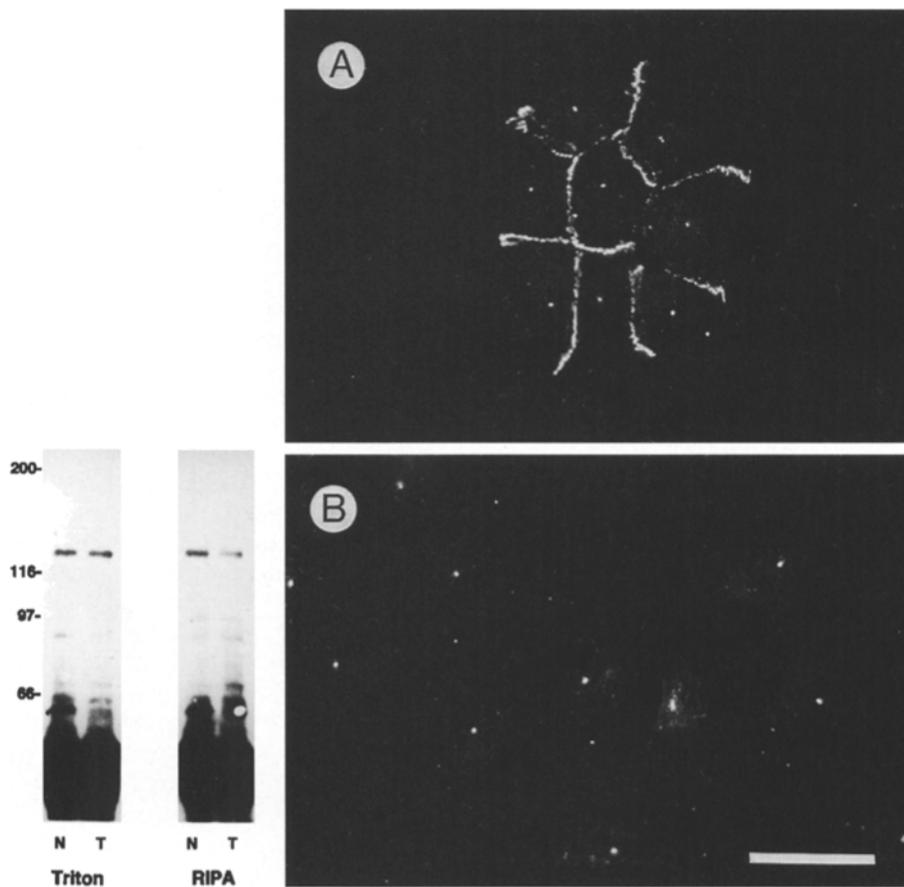


Figure 3. E-cadherin is poorly associated with the actin cytoskeleton of ras-transformed MCF-10A cells. The detergent solubility of E-cadherin was assessed by immunoblotting (*left panel*). Monolayers of normal (*N*) and ras-transformed (*T*) cells were extracted sequentially with Triton and RIPA buffers and blotted with E-cadherin-specific antibodies. Normal (*A*) and ras-transformed (*B*) MCF-10A cells were gently permeabilized with the Triton buffer before fixing with formaldehyde and staining with E-cadherin-specific antibodies (*right panel*). Note the relative absence of cell junction staining in the ras-transformed cells. Bar, 25 μ m.

Lowering the level of tyrosine phosphorylation also restores adherens junctions and leads to a more normal epithelial morphology of the ras-transformed cells.

Materials and Methods

Cell Culture and Antibodies

MCF-10A is a human breast epithelial cell line which originated from a spontaneous immortalization of mammary tissue from a patient with fibrocystic disease (Soule et al., 1990). MCF-10A cells transfected with the normal human Ha-Ras protooncogene or with the mutated (12-V) human Ha-Ras oncogene were obtained from Dr. Bonnie Sloan (Wayne State University, Detroit, MI), and were grown in culture as previously described (Basolo et al., 1991). Monolayers of MCF-10A cells were treated with 875 nM herbimycin A (GIBCO BRL, Gaithersburg, MD) for 18 h at 37°C to inhibit tyrosine kinase activity as described in the text. mAbs specific for E-cadherin (E9), α -catenin (1G5), β -catenin (9F2), and γ -catenin (15F1) were generously provided by Dr. M. J. Wheelock (University of Toledo, Toledo, OH). mAbs to vinculin (Vin 11.5), and E-cadherin (DECMA-1) were purchased from Sigma Chemical Co. (St. Louis, MO). mAbs specific for β -catenin, p120, phosphotyrosine, and E-cadherin were purchased from Transduction Laboratories (Lexington, KY). 9F2 was used for immunofluorescence and immunoprecipitation while the β -catenin antibody (Transduction Laboratories) was used for immunoblotting. Fluorescein-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated anti-phosphotyrosine (PY20) was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA).

Immunofluorescence Microscopy

MCF-10A cells, grown on coverslips for 18 h at 37°C, were fixed for 5 min

with 3.7% formaldehyde in PBS (Sigma Chemical Co.), permeabilized with 0.5% Triton X-100 (Sigma Chemical Co.) in TBS (150 mM NaCl, 50 mM Tris, pH 7.6, 0.1% azide), and stained with primary antibodies for 1 h at 25°C, washed in TBS, and incubated with rhodamine-conjugated goat anti-mouse (Chemicon International, Inc., Temecula, CA) and fluorescein-conjugated goat anti-rabbit (Cappel Laboratories, Durham, NC) when necessary. The coverslips were washed in PBS, rinsed in deionized water, and mounted with Fluor-Save[®] (Calbiochem Corp., La Jolla, CA). Coverslips were viewed on an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY). Fluorescence micrographs were taken on T-max 400 film (Eastman Kodak Co., Rochester, NY).

To assess the distribution of molecules associated with the actin cytoskeleton, MCF-10A cells, grown on coverslips as described above, were permeabilized in 1.0% Triton X-100 in TBS for 2 min, gently washed in TBS for 10 s, and fixed for 5 min in 3.7% formaldehyde in PBS. The samples were then stained with primary and secondary antibodies, mounted, viewed, and photographed.

Preparation of Cell Lysates and Immunoprecipitation

Monolayers of MCF-10A cells were cultured in growth medium to confluency in 100-mm tissue culture dishes (Costar Corp., Cambridge, MA). The monolayers were then extracted sequentially in buffer (150 mM NaCl, 50 mM Tris-Cl, 0.01% NaN₃, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μ g/ml leupeptin, 25 μ g/ml aprotinin) containing 1% Triton X-100 (TX buffer) for 10 min and then in the same buffer containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS (RIPA buffer). After clarification in a microfuge for 5 min at 15,000 rpm, the lysates were immunoprecipitated for 1.5 h at 4°C in the presence of specific antibodies and protein A-Sepharose (Sigma Chemical Co.) conjugated to either rabbit anti-rat or rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as needed. Immunoprecipitated proteins were recovered by centrifugation in a microfuge for 3 min. The immunoprecipitates were then washed four times with the appropriate lysis buffer and resus-

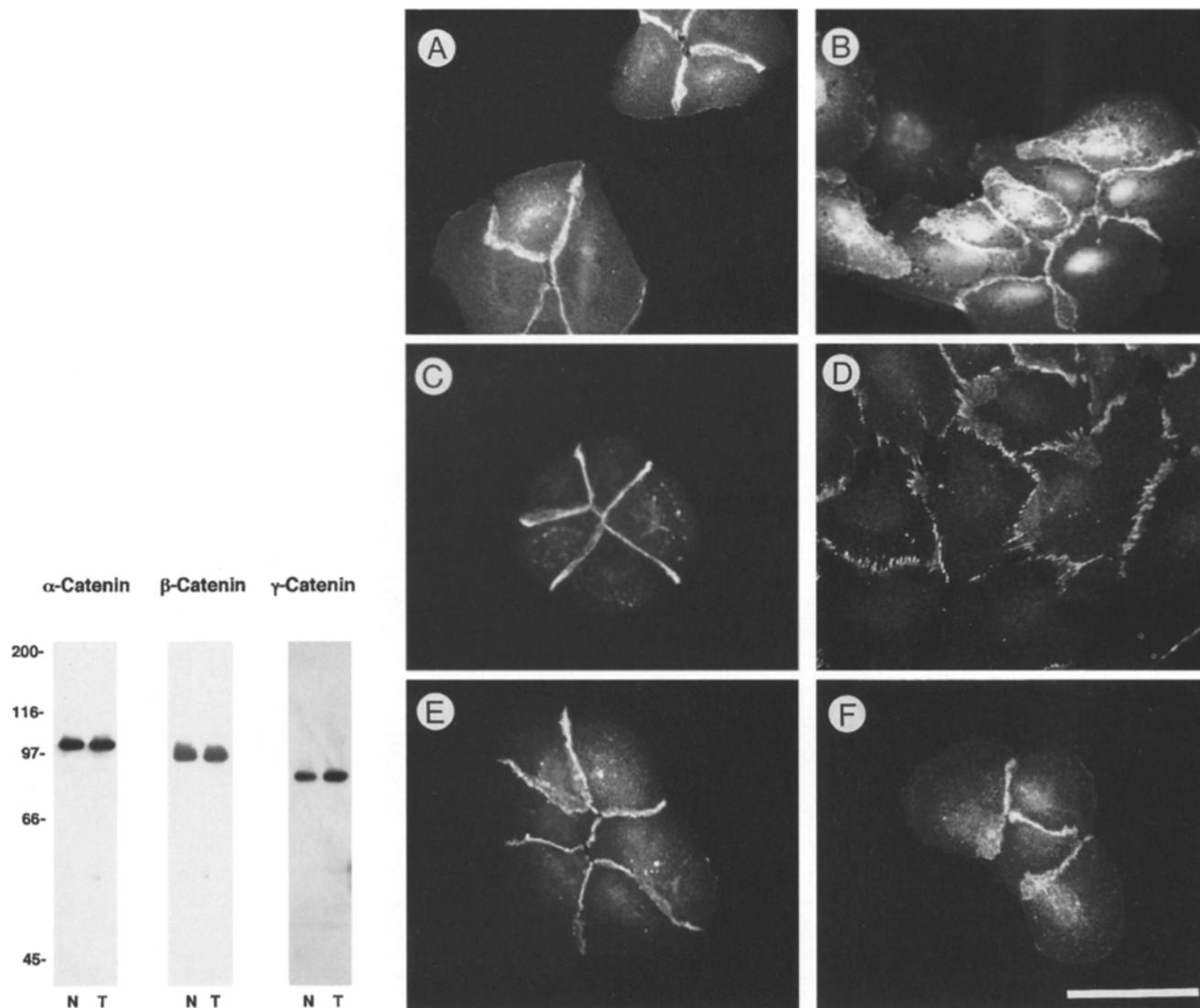


Figure 4. The expression and localization of catenin molecules in normal and ras-transformed MCF-10A cells. (*Left panel*) Cell monolayers dissolved in RIPA buffer were Western blotted with antibodies specific for α -, β -, and γ -catenin as described in the Materials and Methods. (*Right panel*) Normal (A, C, and E) and ras-transformed (B, D, and F) cells were grown in culture overnight and then fixed, permeabilized, and stained with antibodies specific for α -catenin (A and B), β -catenin (C and D), and γ -catenin (E and F). Bar, 25 μ m.

pended in Laemmli SDS gel sample buffer. When indicated, immunoprecipitated samples were denatured by boiling in 1% SDS and reimmunoprecipitated with phosphotyrosine-specific antibodies as recommended by the manufacturer (Transduction Laboratories).

Immunoblotting

Samples were electrophoresed on a 10% SDS-polyacrylamide gel (containing 0.13% bisacrylamide) and transferred overnight to nitrocellulose paper. The blots were blocked in TBSTB (150 mM NaCl, 50 mM Tris-Cl, pH 7.6, 0.05% Tween-20, 1% BSA) containing 2% gelatin (Sigma Chemical Co.), incubated with the appropriate primary antibodies, washed in TBSTB, incubated with the appropriate peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). After washing with TBSTB and TBS for at least 1 h, labeled proteins were detected by enhanced chemiluminescence as recommended by the manufacturer (Amersham Corp., Arlington Heights, IL) on X-OMAT film (Eastman-Kodak Co.). When necessary, the blots were stripped (100 mM 2-mercaptoethanol (Sigma Chemical Co.), 2% SDS, 62.5 mM Tris-HCl, pH 6.7) for

30 min at 60°C. The blots were then washed and reequilibrated in TBS overnight before reprobing with specific antibodies.

Results

Cell Adhesions in Normal and Ras-transformed Epithelial Cells

The architecture of the actin cytoskeleton of the normal and transformed variants of MCF-10A was examined initially by immunofluorescence localization of vinculin and actin. The normal cells displayed an epithelial morphology with vinculin localized at sites of cell-cell contact, typical of cells possessing adherens-type junctions (Fig. 1, A and B). Actin colocalized with vinculin within adhesion belts that surrounded the perimeter of cells within confluent

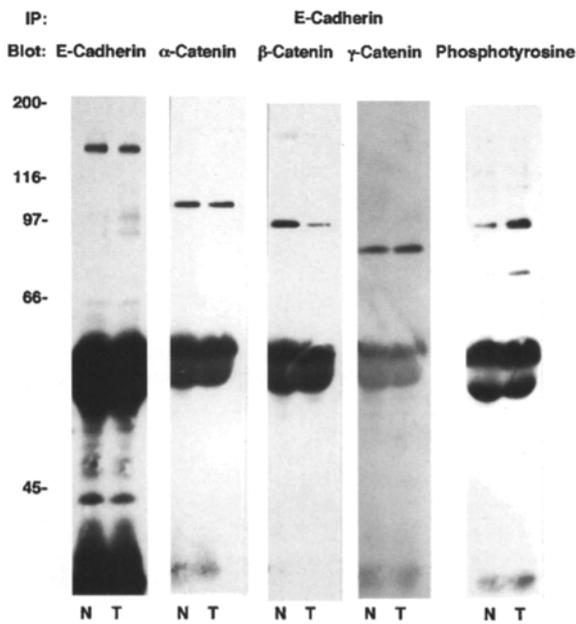


Figure 5. The interaction of β -catenin with E-cadherin is disrupted in the ras-transformed cells. Monolayers of normal (*N*) and ras-transformed (*T*) MCF-10A cells were extracted in RIPA buffer, immunoprecipitated with antibodies specific for E-cadherin, and immunoblotted with antibodies specific for E-cadherin. The blot was then stripped and reprobed with antibodies specific for α -, β -, and γ -catenins and phosphotyrosine-containing molecules.

sheets. Focal adhesions stained with antivinculin close to free, unoccupied edges of colonies. Antivinculin also stained focal adhesions of the normal cells which were plated at low density and thus lacked cell-cell contacts (data not shown). These results indicate that, as with many other epithelial cells, adhesion to neighboring cells promotes the reorganization of the actin cytoskeleton. Stress fibers are replaced by circumferential belts of actin as adherens junctions develop and focal adhesions decrease.

The morphology of the ras-transformed cells was more characteristic of fibroblasts (Fig. 1, *C* and *D*). Neighboring cells were found to be loosely associated. The mesenchymal morphology of transformed cells reflected changes both in the organization of the actin cytoskeleton and the cell junctions. Filamentous actin was observed to be in stress fibers along the basal surface of the transformed cells rather than in the more apical adhesion belts characteristic of the normal cells. Vinculin was no longer found as a tight circumferential band (zonula adherens) but had more the appearance of focal adhesion. Some of the vinculin staining did, indeed, correspond to focal adhesions, co-distributing with talin (data not shown). Interference reflection microscopy confirmed that these sites were focal adhesions (data not shown). Other regions of vinculin staining, however, corresponded to regions of cell-cell contact, but had a jagged, discontinuous appearance compared with the pattern observed in the normal cells (Fig. 1, *C* and *D*).

Recent reports have described decreased E-cadherin expression in a subset of human carcinomas (reviewed in Geiger and Ayalon, 1992; Birchmeier and Birchmeier,

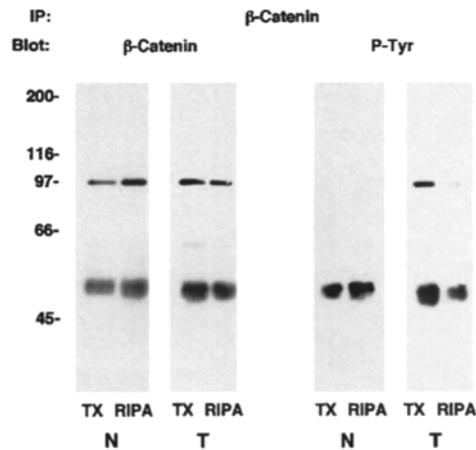


Figure 6. The detergent solubility of β -catenin is altered in ras-transformed cells. Monolayers of normal (*N*) and ras-transformed (*T*) MCF-10A cells were extracted in a mild buffer containing 1.0% Triton X-100 (*TX*) and then in a more vigorous buffer (*RIPA*) containing 1.0% Triton X-100, 0.5% deoxycholate, and 0.1% SDS. The samples were then immunoprecipitated with β -catenin-specific antibodies and blotted with antibodies specific for β -catenin (*left panels*) or phosphotyrosine (*right panels*). Note that tyrosine-phosphorylated β -catenin is present only in the Triton-soluble fraction of cell lysates.

1993). We examined, therefore, whether the expression or distribution of E-cadherin was altered in the transformed cells following transformation with activated ras. Immunoblotting of cell lysates revealed comparable levels of E-cadherin protein in the normal and transformed cells (Fig. 2). However, the distribution of E-cadherin did differ somewhat when examined by immunofluorescence microscopy. Whereas E-cadherin was frequently restricted to a narrow line where adjacent normal cells made contact, the ras-transformed cells displayed broader bands of E-cadherin staining, reflecting regions of cellular overlap. The pattern of E-cadherin staining in the transformed cells did not reveal a jagged, discontinuous pattern comparable to that displayed by vinculin.

The accumulation of E-cadherin at sites of transformed cell contact was somewhat surprising since these cells displayed decreased actin cytoskeletal organization at these cell junctions. Therefore, monolayers of the MCF-10A cells were initially extracted in a mild buffer containing 1% Triton X-100 (*TX* buffer) to solubilize cytosolic molecules. The Triton extraction solubilized most cytosolic proteins. However, the same monolayers were then extracted with a more dissociating buffer (*RIPA* buffer) containing 1% Triton X-100, 0.5% deoxycholate and 0.1% SDS to solubilize proteins associated more tightly with the actin cytoskeleton or those aggregated into Triton-insoluble clusters. Using this strategy, we found that less E-cadherin partitioned into the *RIPA* (Triton-insoluble) fraction from the ras-transformed than normal cells (Fig. 3, *left*). We then examined the localization of detergent resistant E-cadherin molecules by solubilizing molecules with nonionic detergent before fixation. E-cadherin was visualized by staining with specific antibodies (Fig. 3, *right*). In normal cells, E-cadherin staining was preserved at sites of cell-cell contact after detergent extraction for 2 min. Under these

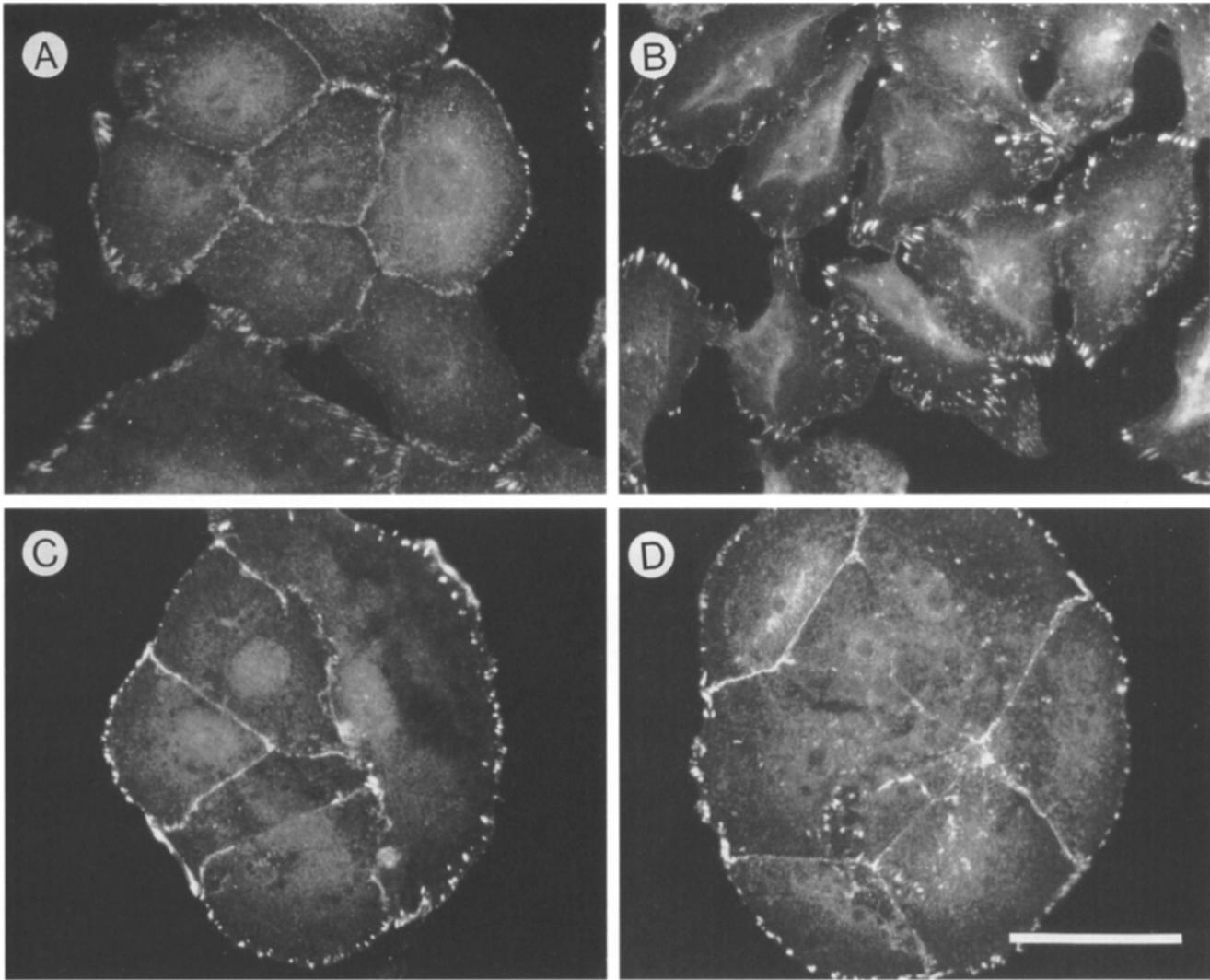


Figure 7. Decreased tyrosine phosphorylation restores adherens junction formation in ras-transformed cells. Normal (A and C) and ras-transformed cells (B and D) were grown on coverslips in culture media overnight and then treated for 18 h in the absence (A and B) or presence (B and D) of the protein tyrosine kinase inhibitor herbimycin A (875 nM). The samples were then stained with vinculin-specific antibodies. Note the restored cell-cell junctions of the herbimycin A-treated cells. Bar, 50 μ m.

extraction conditions relatively little E-cadherin was detected at cell contact sites between transformed cells (Fig. 3).

Normal and Ras-transformed Cells Display Different Cadherin Complexes

We asked whether the increased detergent solubility of E-cadherin in the transformed cells resulted from altered interactions between E-cadherin and the catenin molecules. As the catenins are known to mediate the linkage of cadherins with the actin cytoskeleton (Nagafuchi et al., 1993; Stappert and Kemler, 1993), the expression of α , β , and γ catenin was initially examined. Immunoblotting revealed that catenin molecules were expressed at equivalent levels in the two cell types (Fig. 4), indicating that defective catenin expression was not the cause of the modified adherens junctions of the transformed cells. The subcellular distribution of the catenins was then examined

by immunofluorescence microscopy. These studies revealed that all three catenins were localized to sites of cell-cell contact in both the normal and transformed cells (Fig. 4). With all three antibodies, we were able to detect greater overlapping between adjacent transformed cells. The distribution of α -catenin and γ -catenin was similar to E-cadherin staining of transformed cells. β -catenin, however, was aggregated at more discontinuous sites of cell-cell contact in the transformed cells (Fig. 4), reminiscent of the distribution of vinculin in these cells (Fig. 1). This finding prompted us to examine the association of β -catenin with E-cadherin. Relatively little β -catenin was detected in immunoprecipitates of E-cadherin from the transformed cells (Fig. 5). This decreased β -catenin binding did not represent a general inability of cadherins to interact with catenins as the association of E-cadherin with α -catenin was not altered in the ras-transformed MCF-10A cells and γ -catenin showed a somewhat increased association with

E-cadherin in these cells (Fig. 5). These results indicate that cadherin and catenin molecules appear to be organized into different complexes in the normal and ras-transformed MCF-10A cells.

Tyrosine Phosphorylation of β -Catenin Increases its Detergent Solubility

The detergent solubility of β -catenin was assessed by Western blotting β -catenin immunoprecipitated from cell monolayers extracted sequentially with the TX and RIPA buffers. Most of the β -catenin from the transformed cell monolayer was solubilized by the TX buffer (Fig. 6 A), whereas the TX buffer extracted about half of the β -catenin from monolayers of normal cells. The remaining β -catenin in the normal cells was solubilized by the subsequent, more vigorous extraction with RIPA buffer. β -catenin has recently been shown to act as a substrate for tyrosine kinases and this tyrosine phosphorylation correlates with the disassembly of adherens junctions (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Shibamoto et al., 1994). We examined the phosphotyrosine content of β -catenin in the two cell types (Fig. 6 B). Tyrosine-phosphorylated β -catenin could be detected in the Triton-soluble fraction of both cell types. Little, if any, tyrosine-phosphorylated β -catenin could be detected in the RIPA fraction from either the normal or ras-transformed cells.

The correlation of tyrosine phosphorylation of β -catenin with its solubility was examined further by treating monolayers of MCF-10A cells with the protein tyrosine kinase inhibitor herbimycin A. Herbimycin A induced morphological changes in the transformed cells (Fig. 7). Whereas the untreated transformed cells maintained a more mesenchymal morphology (Fig. 7 B), after herbimycin A treatment they displayed a more epithelial morphology and were tightly associated with adjoining cells (Fig. 7 D). Staining with antivinculin revealed that much of the vinculin in the untreated cells was enriched within focal adhesions. After herbimycin A treatment, the vinculin staining was mostly restricted to sites of cell-cell rather than cell-substrate adhesion. Decreasing the level of tyrosine phosphorylation thus restores the normal morphology and interactions of the ras-transformed MCF-10A cells.

Treatment with herbimycin A decreased the tyrosine phosphorylation of many proteins (Fig. 8), one of which was β -catenin (Fig. 9). Immunoprecipitation of β -catenin from lysates of herbimycin A-treated cells indicated that relatively little β -catenin was solubilized by the TX buffer in contrast to the untreated cells. Most of the β -catenin could only be solubilized from the treated cells by the RIPA buffer. When the same blot was stripped and re-probed with antiphosphotyrosine, tyrosine-phosphorylated β -catenin was only detected in the Triton-soluble fraction of the untreated cells (Fig. 9). Herbimycin A treatment of the transformed cells also restored the association of β -catenin with E-cadherin, but did not affect the binding of α -catenin to E-cadherin (Fig. 9).

Tyrosine Phosphorylation of p120 Cas Increases E-cadherin Association

The experiments above suggest that the elevated tyrosine phosphorylation of β -catenin in ras-transformed MCF-

10A cells results in decreased E-cadherin binding. Yet blotting with antiphosphotyrosine revealed a 97-kD tyrosine-phosphorylated protein in association with E-cadherin in the transformed cells (Fig. 5). It was unlikely that this represented β -catenin since tyrosine phosphorylation of β -catenin decreased its association with E-cadherin. In the course of a survey of tyrosine phosphorylated proteins that might function in focal adhesions or cell-cell adherens junctions in these ras-transformed MCF-10A cells, we found elevated phosphotyrosine in p120 Cas (Fig. 10). This molecule recently has been shown to interact with cadherins (Reynolds et al., 1994). Indeed, p120 Cas was enriched within the cell-cell junctions of both the normal and ras-transformed MCF-10A cells (Fig. 11) and p120 Cas immunoprecipitated with E-cadherin from the ras-transformed, but not normal MCF-10A cells (Fig. 10). The converse experiment also revealed p120 Cas associated with E-cadherin immunoprecipitated from the ras-transformed cells (Fig. 10). To determine whether tyrosine-phosphorylated p120 Cas was capable of interactions with E-cadherin, immunoprecipitated E-cadherin complexes were dissociated by boiling in detergent and reimmunoprecipitated with antiphosphotyrosine. Immunoblotting revealed the presence of p120 Cas in this fraction, indicating that tyrosine-phosphorylated Cas associated with E-cadherin from the transformed, but not the normal MCF-10A cells (Fig 10, right).

Interactions between Cas and E-cadherin might contribute to the increased detergent solubility of E-cadherin at the cell-cell junctions of the ras-transformed MCF-10A cells. Unlike β -catenin, virtually all of the Cas in both cell types could be extracted with the TX buffer (Fig. 12). In both the normal and transformed MCF-10A cells, Cas exists primarily as a 97-kD isoform. β -catenin is also a 97-kD protein that displays elevated tyrosine phosphorylation in the transformed cells. To examine the possibility that the p120 Cas antibodies used in our studies might have cross-reacted with β -catenin, lysates from the normal and transformed cells were immunoprecipitated with anti-Cas, and blotted with β -catenin-specific antibodies (Fig. 12). We did not detect β -catenin in material precipitated by the

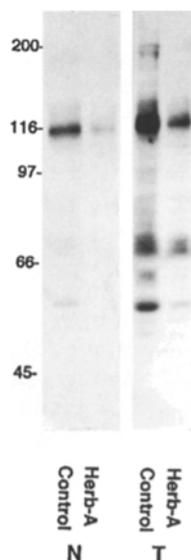


Figure 8. Tyrosine-phosphorylated proteins in MCF-10A cells. RIPA extracts of monolayers of normal (N) and ras-transformed (T) cells treated in the absence (Control) or presence (Herb-A) of 875 nM herbimycin A (18 h, 37°C) were blotted with phosphotyrosine-specific antibodies.

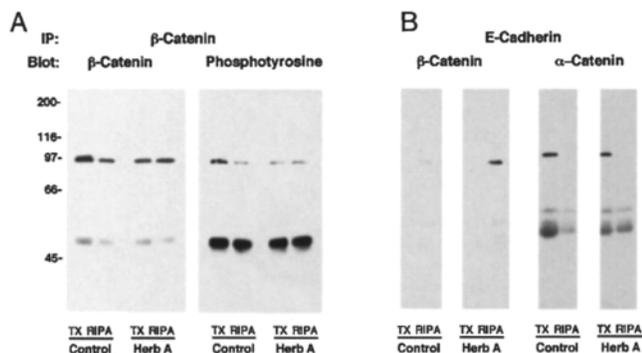


Figure 9. Tyrosine kinase inhibitors restore the interactions of β -catenin in ras-transformed cells. Monolayers of ras-transformed cells were treated in the absence (*Control*) or presence (*Herb A*) of the tyrosine kinase inhibitor herbimycin A (875 nM, 18 h, 37°C). The monolayers were then extracted and immunoprecipitated with antibodies for either β -catenin (*A*) or E-cadherin (*B*). The samples were Western blotted with antibodies specific for β -catenin, phosphotyrosine, or α -catenin.

anti-Cas, indicating not only that the antibodies did not cross-react, but that p120 Cas and β -catenin molecules do not interact strongly in the MCF-10A cells.

Discussion

Transformation of MCF-10A breast epithelial cells with an activated form of ras alters the phenotype of the cells. Morphologically, they appear more fibroblastic; they reveal increased numbers of focal adhesions and altered cell–cell adhesions. The ras-transformed MCF-10A cells have elevated phosphotyrosine in many proteins and this appears to be a critical factor in the morphological transformation since it can be reversed by tyrosine kinase inhibitors that depress the level of tyrosine phosphorylation. This does not appear to be a clonal effect of this cell line, because bulk cultures of MCF-10A cells recently transformed with activated ras (as well as with other ras-related GTP-binding proteins) display similar elevation in tyrosine phosphorylation and similarly altered cell–cell junctions (M. S. Kinch, unpublished observations).

One of the conclusions suggested by this current study is that several types of cadherin–catenin complexes may coexist within the same cell. Recent studies have concluded that β - and γ -catenin can be complexed with E-cadherin within mutually exclusive complexes (Hinck et al., 1994; Näthke et al., 1994). Therefore, the normal MCF-10A cells likely assemble at least two E-cadherin complexes which contain either β - or γ -catenin. In the ras-transformed cells, however, E-cadherin can be detected in complex with γ -catenin, but not with β -catenin. Instead, we have found E-cadherin complexes containing p120 Cas in the ras-transformed cells. The identification of p120 Cas as yet another cadherin-associated protein increases the complexity of E-cadherin interactions and raises the question as to whether p120 Cas and β -catenin are also found within mutually exclusive complexes. Comparisons between the normal and ras-transformed MCF-10A cells appear to support this idea. A different conclusion, however, was reached in

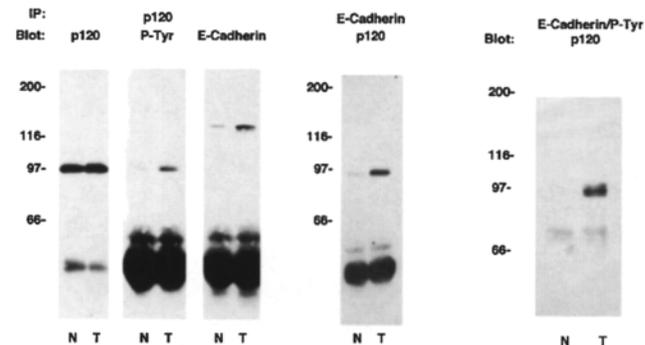


Figure 10. E-cadherin and p120 interact in ras-transformed MCF-10A cells. Monolayers of normal (*N*) and transformed (*T*) MCF-10A cells were extracted and immunoprecipitated with antibodies for p120 or E-cadherin. The left three panels show the same blot which has been stripped and reprobed with the appropriate antibody. The middle panel shows immunoprecipitated E-cadherin complexes blotted with anti-p120 Cas. Note that control experiments can be found in Fig. 5. The right panel shows isolated E-cadherin complexes that were dissociated by boiling in a buffer containing 1% SDS and reimmunoprecipitated with antiphosphotyrosine. The samples were then resolved by SDS-PAGE and blotted with antibodies for p120.

another study, in which both p120 Cas and β -catenin were found in association with immunoprecipitated E-cadherin (Reynolds et al., 1994). A possible explanation for the difference in these results may be in the behavior of the different p120 Cas isoforms expressed in these different cells.

A further level of cell junction complexity is suggested by the observation that N-cadherin can be found at the discontinuous cell–cell junctions of the ras-transformed, but not normal MCF-10A cells (our unpublished data). Thus, at least two different cadherins (i.e., E- and N-cadherin) and three different catenins (i.e., β -catenin, γ -catenin, and p120 Cas) may participate in distinct junctional complexes in the MCF-10A epithelial cells. Assuming all combinations of these molecules can be organized into functional units, it is possible that at least six different cadherin complexes could be present and contribute to different junctional properties.

In the ras-transformed cells, β -catenin displays increased tyrosine phosphorylation and this correlates inversely with cadherin binding. In contrast, elevated tyrosine phosphorylation of p120 Cas in the ras-transformed cells correlates with its increased association with E-cadherin. It seems likely that the tyrosine phosphorylation of these proteins and their altered interactions with E-cadherin affect the stability of the adherens junctions in the ras-transformed cells. However, in these cells, multiple proteins reveal increased tyrosine phosphorylation and it will be important in the future to determine whether any of these or other factors contribute to the changes in cell–cell interaction. As β -catenin and p120 Cas share some sequence homology (Reynolds et al., 1992; Peifer et al., 1994), it is tempting to speculate that they compete for cadherin binding and that the level of tyrosine phosphorylation determines the outcome of this competition. Under conditions of low tyrosine phosphorylation, cadherin binding to β -catenin may be favored and promote the recruitment of the actin cy-

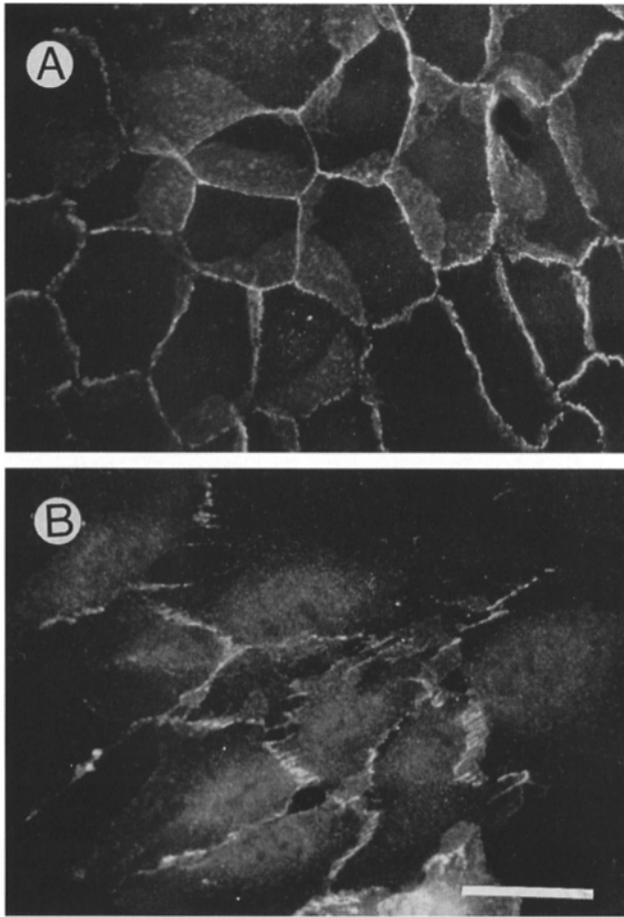


Figure 11. Immunolocalization of p120 Cas in MCF-10A cells. Normal (A) and ras-transformed (B) MCF-10A cells were grown on coverslips in culture media overnight and stained with p120 Cas-specific antibodies. Bar, 25 μ m.

toskeleton into classical epithelial adherens-type junctions. Upon elevated tyrosine phosphorylation, p120 Cas might displace β -catenin for cadherin binding. Based on its greater solubility in buffers containing Triton X-100, we suspect that p120 Cas interacts more weakly with the actin cytoskeleton than β -catenin. Under conditions in which β -catenin is displaced by p120 Cas, the weaker association of p120 Cas with the actin cytoskeleton should promote the formation of more labile cell-cell adhesions, thereby contributing to the fibroblastic morphology of these transformed epithelia. One question raised by these studies is why p120 Cas and β -catenin are not found in the E-cadherin complexes of the normal and transformed cells, respectively, and yet in these cells are localized to sites of cell-cell contact. One possible explanation is that other interactions localize these molecules in the absence of their binding to E-cadherin. Indeed, the localization of β -catenin between ras-transformed cells corresponds with increased interactions with other cadherins and with increased association of APC, which colocalizes with β -catenin at sites of cell-cell contact (M. S. Kinch, unpublished observations). Alternatively, the procedures used to isolate E-cadherin complexes might have abrogated lower affinity interactions. It will be of interest to examine further the

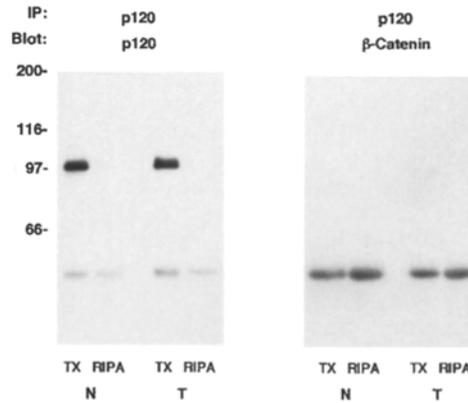


Figure 12. p120 Cas is detergent soluble and does not interact with β -catenin. The distribution of p120 Cas in the Triton (TX) and RIPA detergent fractions was assessed by Western blotting immunoprecipitated p120 Cas complexes with anti-p120 (left panel). The same blots were then stripped and reprobed with β -catenin-specific antibodies (right panel).

interactions of β -catenin and p120 Cas to distinguish between these possibilities.

There are numerous examples in vitro where tyrosine phosphorylation regulates the stability of cell-cell and cell-substrate junctions. The addition of growth factors such as EGF or hepatocyte growth factor, scatter factor (HGF/SF) rapidly dissociate colonies of normal epithelia (Stoker et al., 1987; Weidner et al., 1991; Shibamoto et al., 1994). This "scattering" of the epithelia is accompanied by increased motility and a mesenchymal morphology, similar to our observations with ras-transformed cells. Tyrosine phosphorylation has been implicated in mediating these effects as the receptors for these growth factors are themselves protein tyrosine kinases that display elevated activity after ligand binding. β - and γ -catenins (plakoglobin) reveal elevated tyrosine phosphorylation in cells scattered by these growth factors (Shibamoto et al., 1994). Moreover, β -catenin seems capable of direct interactions with the EGF receptor, and tyrosine phosphorylation of β -catenin in EGF treated cells corresponds with detergent solubility (Hoschuetzky et al., 1995). A similar loss of epithelial cell phenotype and functional inactivation of cadherin-mediated cell adhesion can be induced by direct elevation of tyrosine phosphorylation, for example by transformation with v-src or inhibition of protein tyrosine phosphatase activity with vanadate (Volberg et al., 1991; Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Reynolds et al., 1994). With elevated tyrosine phosphorylation there is a rapid reorganization of the actin cytoskeleton and enhanced formation of focal adhesions (Volberg et al., 1992). In all of these situations, there is a transition from an epithelial to a more mesenchymal morphology that is associated with elevated tyrosine phosphorylation.

In general, ras is thought to act downstream of tyrosine kinases, such as the receptors for many growth factors and upstream of a cascade of serine/threonine kinases (reviewed in Khosravi-Far and Der, 1994). It will be important to identify the tyrosine kinases acting on these junctional proteins in the ras-transformed cells. One possibility

is that the ras-transformed cells are releasing growth factors that are stimulating tyrosine phosphorylation through an autocrine mechanism. Indeed, ras-transformed MCF-10A cells display increased levels of amphiregulin, a member of the EGF family (Normanno et al., 1994). Since activation of ras is a common event in many human tumors of epithelial origin (reviewed in Clark and Der, 1994), the elevated tyrosine phosphorylation and the resulting altered adhesive interactions and cytoskeletal organization likely contribute to the invasive and metastatic properties of these tumors.

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